See also: **II/Crystallization:** Additives: Molecular Design; Control of Crystallizers and Dynamic Behaviour; Polymorphism. **III/Supercritical Fluid Crystallization.**

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Electrophoresis

M. J. Schmerr, National Animal Disease Center, Ames, IA, USA

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Transmissible spongiform encephalopathies are neurodegenerative diseases found in both humans and animals. The oldest known member of this family of diseases is scrapie in sheep and goats and the most infamous member is bovine spongiform encephalopathy or 'mad cow disease'. These diseases are fatal for the individuals who become infected. As a result, there is a considerable amount of interest in developing methods to detect early infection. This would enable removal of animals from food chains and by-products used for cosmetic and health care. For humans, an early diagnosis may make it possible to treat infected individuals with drugs to arrest the course of the disease.

A feature of these diseases is the accumulation of rod-shaped fibrils in the brain that form from an aggregated protein. This abnormal protein is a protease-resistant form of a normal host cell glycoprotein (prion protein). When the aggregated protein is denatured in sodium dodecyl sulphate (SDS) and β-mercaptoethanol, a monomer form of $M_r \sim 27$ kDa is observed. This abnormal prion protein is used as a marker for infection with a transmissible spongiform encephalopathy.

The abnormal prion protein is insoluble in most biological buffers, whereas the normal prion protein is soluble. In natural infections, the abnormal prion protein is found in very low concentrations. It is found in higher amounts in rodent-adapted strains of the disease. These properties of insolubility and low concentrations present quite a challenge for the development of analytical methods to detect this protein.

Most of the methods used to detect the prion protein are based on histological techniques and are used postmortem. Immunoassays can be used to measure the prion protein. Most of the antibodies that have been produced to the abnormal prion protein have been made to the denatured form. Removing these denaturants is a major problem in the development of immunoassays. Western blot can be used to detect the prion protein but cannot be easily automated. Some new approaches using chemiluminescence and time-resolved fluorescence in plate assays have been developed. The amount of prion protein detected by these assays is in the range of picomoles or > 500 fmol. To improve sensitivity, we have approached this problem using capillary electrophoresis with laser-induced fluorescence. Fluorescent-labelled peptides from immunogenic epitopes of the prion protein can be detected in the attomole range using this technique. A competition immunoassay using fluorescein-labelled peptides was developed which is

able to detect the abnormal prion protein in the low picogram range; this method can quantitate the amount of prion protein. This assay was based on the separation of the free peptide from the immunocomplexed peptide. Unlike most immunoassays which measure only the antibody-bound ligand, both the free and the bound peptide can be measured.

Method Development

Preparation of Tissues

Scrapie-infected tissues including brain, lymph node and buffy coats were obtained from sheep confirmed positive for abnormal prion protein by Western blot. Normal tissues were obtained from sheep from a scrapie-free flock and were confirmed negative by the above tests. Briefly, the tissues were weighed, and ground to a fine powder in liquid nitrogen. Buffy coats were prepared from blood and placed in 2 mL of 20 mmol L^{-1} phosphate pH 7.0, 0.15 mol L^{-1} NaCl, and frozen at $-70°C$ until they were processed as the tissues. After grinding, the tissues were placed in 20 mmol L^{-1} Tris pH 7.4, 0.15 mol L^{-1} NaCl, 0.005 mol L^{-1} $MgCl_2$ (10% w/v) and incubated at 37°C for 1 h in 50 μg mL^{-1} DNase. After incubation with DNase the tissue homogenates were treated with proteinase K (50 μg mL^{-1}) for 1 h at 37°C and held overnight at 4°C. Sodium *N*-lauroylsarcosine was added to the homogenate to make the solution 10% in the detergent. The homogenate was incubated for 1 h at 37°C and then was centrifuged at 10 000 g for 20 min to remove particulates. The resultant supernatant fluid was centrifuged at 230 000 **g** for 1 h. The pellet was resuspended in 10 mmol L^{-1} Tris pH 7.4 (250 μL g^{-1} of the initial brain sample). The sample was solubilized in 0.01 mmol L^{-1} Tris HCl, pH 8 containing 2 mmol L^{-1} ethylenediaminetetraacetic acid 5% SDS and 10% hexafluoro-2-propanol at 100°C for 10 min.

Chromatography

To remove the SDS, the sample was applied to a poly-HYDROXYETHYL A (PolyLC, Inc., Columbia, MD, USA) high performance liquid chromatography column (200 × 4.6 mm) in 95% acetonitrile, 5% water containing 0.1% trifluoroacetic acid and 50 mmol L^{-1} hexafluoro-2-propanol (buffer A). The flow rate was 0.5 mL min^{-1}. The conditions for eluting abnormal prion protein were buffer A for 8 min and then a linear gradient to 100% water containing 0.1% trifluoracetic acid and 50 mmol L^{-1} hexafluoro-2-propanol (buffer B) in 15 min, 100% buffer B for 10 min. Peak fractions were collected and dried in a vacuum centrifuge. Fractions were resuspended in 10 μL of dH_2O and tested for abnormal prion protein in the capillary electrophoresis assay.

Peptide Synthesis and Antibody Preparation

Four peptides from the prion protein were synthesized. The peptide sequences were CGQGGGTHNQWNKPSL (spanning amino acid positions 89–103), CNDWEDRYYRENMYR (142–154), (CRYPNQVYYRPVDRYSNQNNFVHD (155–177) and RESQAYYQRGASVIL (218–232) (Multiple Peptide Systems, San Diego, CA, USA). The peptides were labelled with fluorescein through a γ-butyric acid linkage on the N-terminus during synthesis. The peptide 218–232 is used here as a representative sample.

Rabbits were immunized with each peptide and specific antibodies were produced for each peptide. These antiserums reacted with scrapie-infected brain but not with normal brain on Western blot analysis. Rabbit IgG was prepared by passing each antiserum over an affinity column. Briefly, 10 mg of a peptide was coupled to agarose resin modified with an *N*-hydroxyl succinimide ester in 1.0 mL dimethyl formamide at room temperature for 20 min. After coupling, the resin was washed with 5 mL of 0.1 mol L^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.5 (column wash buffer). Unreacted ester groups were deactivated with 0.1 mol L^{-1} N-(2-hydroxyethyl)piperazine-N′-(4-butanesulfonic acid) (HEPBS) pH 8.0 and 0.1 mol L^{-1} NH_4Cl for 15 min. Before antibodies were applied to the peptide column they were purified using protein G chromatography. After diluting 1 : 2 in column wash buffer, the antibodies were then applied to the affinity column and recycled over the column for several cycles. The column was washed with column wash buffer and the antibodies eluted with 0.1 mol L^{-1} NaH_2PO_4, pH 2.5 into tubes containing 50 μL of 1 mol L^{-1} HEPBS, pH 8.5. The absorbance was measured at 280 nm. Fractions with absorption at 280 nm were pooled, aliquoted and frozen at $-20°C$.

Immunocomplex Formation

Fifteen microlitres containing ~0.2–20 pmol of the fluorescent-labelled peptide were mixed with varying amounts (0.5–5 μg) of purified rabbit IgG to determine the antibody concentration that forms ~50% of the total immune complex formation. The final volume of the sample was adjusted to 20 μL with capillary running buffer. After mixing the components, the samples were incubated at 25°C for ~10 min and at 4°C overnight. The height of the immune complex peak was measured and replicate samples of the peaks varied less than 1%. Slight changes in the antibody reactivity, temperature and

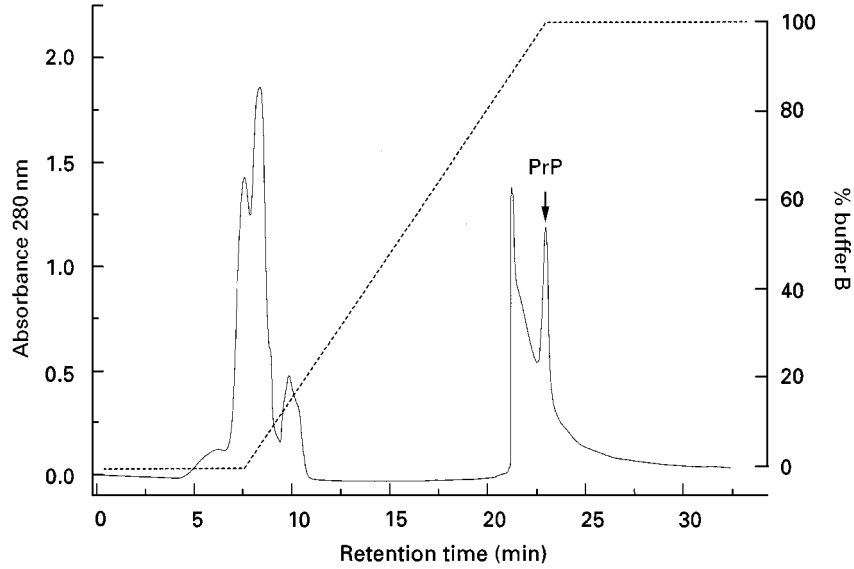

Figure 1 A chromatogram showing hydrophilic interaction chromatography on poly-HYDROXETHYL A. The peak that was positive for abnormal prion protein (PrP) is indicated. The gradient conditions for running the column are shown by the dashed line.

preparation of the running buffer caused small variations in the height of the immunocomplex peak from day to day. Known concentrations of unlabelled peptides corresponding to the fluorescent-labelled peptides were used to generate standard curves.

Free Zone Capillary Electrophoresis

Free zone capillary electrophoresis was performed on a Beckman P/ACE 5500 controlled by P/ACE Station software. Laser-induced fluorescence detection was done using an air-cooled argon laser with excitation at 488 nm and emission at 520 nm. An unmodified capillary 20 cm (length to the detector) $\times$ 20 μm i.d., total length 27 cm capillary was used with a 200 mmol L^{-1} Tricine buffer that was adjusted to pH 8.0 by 6 mol L^{-1} NaOH. This buffer was selected after studying the effect of higher pHs and other concentrations of the buffer on the separation, immunocomplex formation and fluorescence. To prevent the abnormal prion protein from adhering to the

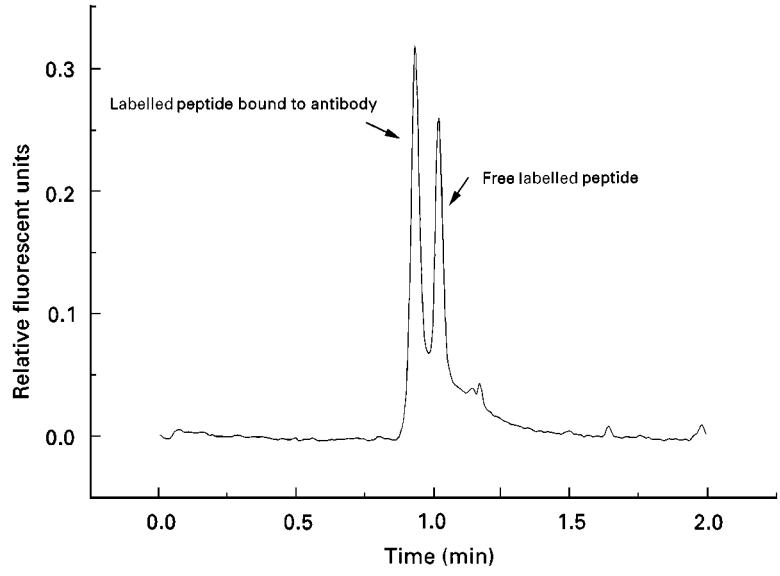

Figure 2 An electropherogram showing the immunocomplex peak for the fluorescein-labelled peptide 218–232 and the free peptide peak.

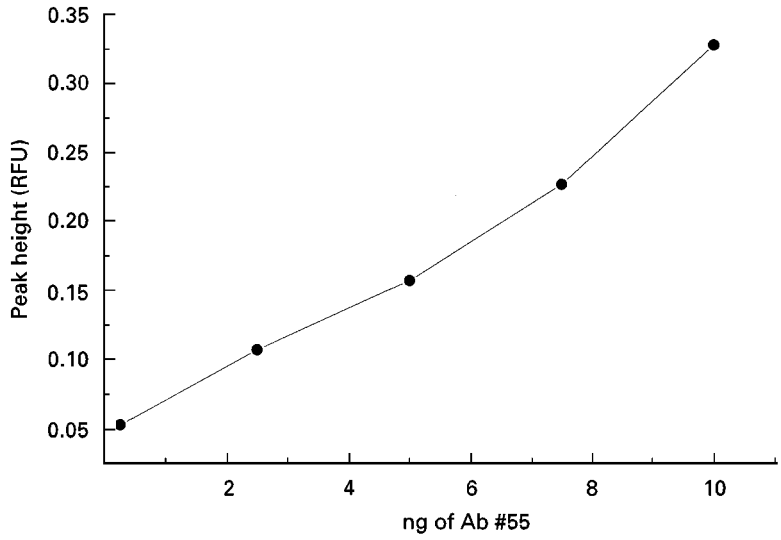

Figure 3 A plot of the peak height of the immunocomplex peak versus the amount of antibody added to the assay.

capillary walls, 0.1% *n*-octylglucoside (Boehringer Mannheim, Indianapolis, IN, USA) and 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) were added to the buffer. In preparation for separation, the capillary was rinsed for 1 min with 0.25 mol L^{-1} NaOH, rinsed for 2 min with water and then rinsed for 2 min with buffer. The separating conditions were 30 kV for 3 min at 20°C with a current of ~ 20 μA. The sample was injected for 15 s followed by a 5 s injection of running buffer. The sample volume was ~ 0.95 nL. Rinses were carried out under high pressure and sample injection carried out under low pressure.

Results

Hydrophilic Interaction Chromatography

Figure 1 shows a chromatogram of the results of hydrophilic interaction chromatography. This chromatography removes the SDS and other interfering compound so that the reproducibility of the assay is improved.

Capillary Electrophoresis Immunoassay

By the addition of fluorescein at the amino terminal during synthesis, the sensitivity of this assay is enhanced 100-fold relative to the chemical addition of

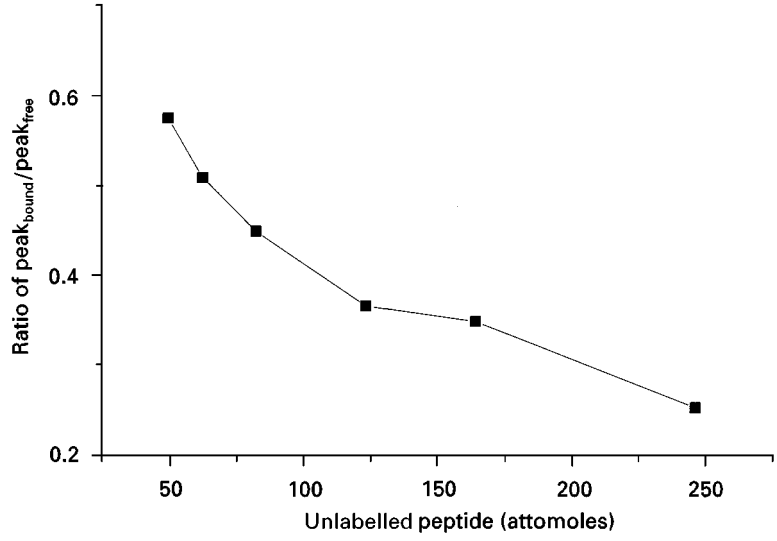

Figure 4 Plot of the ratio of the height of the immunocomplex peak/height of the free peptide peak versus the amount of unlabelled peptide added to the assay.

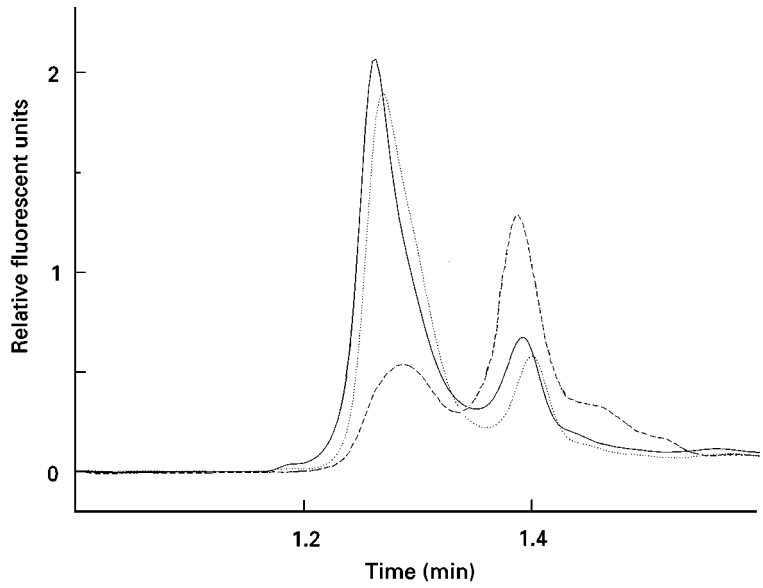

Figure 5 Representative electropherograms of antibody control (continuous line), normal brain sample (dotted line) and scrapie-infected brain sample (dashed line).

fluorescein after synthesis of the peptide. An electropherogram showing the peptide and the immunocomplex peak when antibody is added to the assay is shown in **Figure 2**. A titration curve of antibody amount versus the height of the immunocomplex peak is shown in **Figure 3**. The amount of antibody that binds ~50% of the fluorescein-labelled peptide was chosen as the amount to be used in the competition assays of the abnormal prion protein with the labelled peptide for binding sites on the specific antibody. Competition is determined by measuring the ratio of the height of the immunocomplex peak and of the free peptide peak. A standard curve was determined by adding known amounts of unlabelled peptide into the assay. This curve is shown in **Figure 4**. Electropherograms representing the immunocomplex peak, a preparation from a normal sheep and a preparation from a scrapie-infected sheep are shown in **Figure 5** (peptide 218–232). An electropherogram representing a sample from a lymph node of an infected sheep is shown in **Figure 6**. **Figure 7** depicts three electropherograms showing the antibody control,

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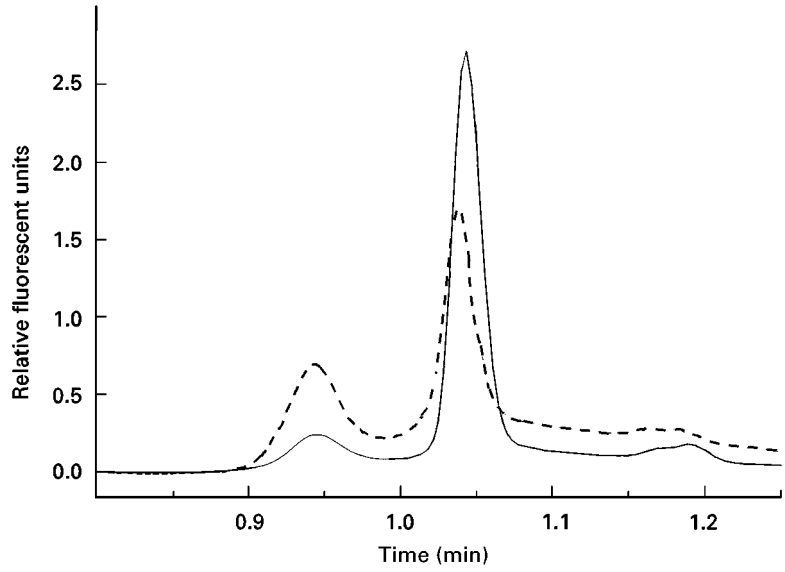

Figure 6 Representative electropherograms of antibody control (dashed line) and a sample from an infected lymph node (continuous line).

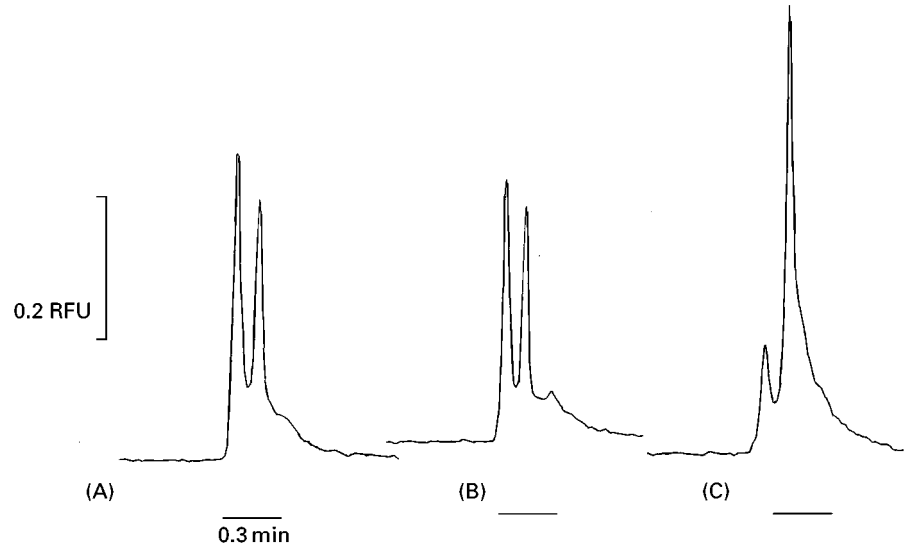

Figure 7 Representative electropherograms of (A) antibody control; (B) buffy coat from a scrapie-negative sheep; (C) buffy coat from a scrapie-positive sheep.

samples extracted from buffy coats of a normal sheep and from a buffy coat of a scrapie-infected sheep.

Concluding Remarks

The capillary electrophoresis assay described in this study is reproducible, more sensitive and faster than other analytical tests. The samples used in the capillary electrophoresis assay were obtained from brain and the lymphoid system of the animals. The sensitivity of this assay made it possible to test samples from other tissues that contain much less abnormal prion protein than brain samples. This assay has the potential to use tissues and fluids from live animals and diagnose animals prior to the onset of clinical signs of disease. Automation of this test could lead to more economical and efficient methods for testing for abnormal prion protein.

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Field Flow Fractionation

R. Hecker and H. Cölfen, Max-Planck-Institut für Kolloid und Grenzflächenforschung (Kolloidchemie), Am Mühlenberg, Golm, Germany

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Introduction

This review focuses on the use of field-flow fractionation (FFF) for the characterization of proteins and protein assemblies such as protein aggregates, DNA